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TISSUE SPECIFIC PROMOTERS

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The present invention provides a genetic construct providing preferential expression in specific tissue of a coding sequence. The construct includes the following elements (1) a steroid responsive promoter from a tissue specific gene, (2) a coding sequence, and (3) an SV40 enhancer. The elements are arranged as follows: the steroid responsive promoter from the tissue specific gene being upstream of the coding sequence and the SV40 enhancer being 3' of the coding sequence, or the SV40 enhancer being upstream of the coding sequence and the steroid responsive promoter from the tissue specific gene being positioned between the SV40 enhancer and the coding sequence, or the steroid responsive promoter from the tissue specific gene being upstream of the coding sequence and the SV40 enhancer being positioned within an intron within the coding sequence. The promoter has a lower level of steroid responsiveness in the construct than in its native state.

TISSUE SPECIFIC PROMOTERS

Description of correspondent: **WO9833903**

Tissue Specific Promoters

FIELD OF THE INVENTION

The present invention is concerned with combinations of prostate gene promoters with a general enhancer, the SV40 enhancer, giving rise to essentially androgen-independent promoters active in prostate cells which are androgen-independent in their growth and survival.

BACKGROUND OF THE INVENTION

A number of genes have been identified which are expressed predominantly in the prostate in males. Promoters : regulatory regions of a number of such genes have been studied, either using transfection techniques or by follow gene expression in transgenic mice. Most of the genes identified as prostate-specific are androgen-inducible and this aspect of their function has been studied in some detail. Thus the importance of androgen response element for induced expression and/or binding of androgen receptor have been characterised in the prostate specific antigen (PSA) (Cleutjens, Vaneekelen et al. 1996; Riegman, Vliestra et al. 1991), human glandular kallikrein (KLK2) (Mur Tindall et al. 1993), rat prostatic steroid binding proteins (PSBP) (Claessens, Rushmere et al. 1990; Rushmere, Parker et al. 1987), probasin (Pb) (Kasper, Rennie et al. 1994; Rennie, Bruchofsky et al. 1993) and prostatic acid phosphatase genes (Virkkunen, Hedberg et al. 1994) and in regulatory elements in the introns of the rat PSBP C3(1) gene (Celis, Claessens et al. 1993) and the rat 20-KDa androgen regulated protein (Ho, Marschke et al. 1993).

Elements involved in conferring prostate-specificity of expression per se, as distinct from androgen responsiveness have not been well characterised, though tissue-specific factors binding to regions of the PSBP C3 gene promoter and 1st intron have been identified (Celis, Claessens et al.

1993; Zhang, Parker et al. 1990). The gene for rat PSBP C(3) with 4 kb upstream and 2 kb downstream flanking sequences is expressed tissuespecifically and with appropriate hormonal control in transgenic mice (Allison, Zhai et al. 1989). The use of a 5kb upstream region from the rat PSBP C3(1) gene to express the SV40 T-antigen could elicit prostate tumours, but expression was not highly restricted and other abnormalities were common (Maroulakou, Anver et al. 1994). Studies with transgenic mice have established that regions of the probasin and PSBP C(3) genes can confer prostate specificity.

The PSA and probasin regulatory regions are the two most studied among prostate-expressed genes. It has been established that a 430 bp region upstream of the rat probasin gene is able to confer prostate specificity of expression on a reporter gene (Greenberg, Demayo et al. 1994; Matusik, WO9403594); when used to target expression of the SV40 T-antigen, prostate tumours develop specifically (Greenberg, Demayo et al. 1995). This expression is not totally specific but specificity is significantly improved by inclusion of MAR (matrix attachment regions) from the chick lysozyme gene (Greenberg, Demayo et al. 1994). The 430 bp promoter region is strongly responsive to androgen induction and androgen response elements which bind the androgen receptor (AR) have been characterised (Claessens, Rushmere et al. 1990; Kasper, Rennie et al. 1994; Matusik, WO9403594; Rennie, Bruchofsky et al. 1993). A negative regulatory region between bases -426 and -286 has also been identified (Rennie, Bruchofsky et al. 1993).

The PSA upstream region (to -630 bp) also acts as a strongly androgen responsive promoter and androgen response elements have also been characterised. This region is not sufficient to direct tissue specific expression in transgenic mice, however. Use of the 630 bp human PSA promoter region to express an activated Ha-ras oncogene in transgenic mice led to the development of salivary gland and not prostate tumours (Schaffner, Barrios et al. 1994). An enhancer region has recently been identified in the region 4 to 5kb upstream of the transcription start site. The PSA enhancer has been shown to act as an androgen-inducible enhancer and in combination with the PSA promoter to display significant cell-type specificity (Henderson, WO9519434; Schuur, Henderson et al. 1996). Als Pang et al. have reported that the equivalent promoter region isolated from a prostate cancer patient contained 7 mutations compared to the published sequence and was highly active in the prostate cancer cell line LNCaP

(Beldegrun and Pang, WO9614875; Pang, Taneja et al. 1995).

The viral SV40 enhancer was the first enhancer identified and it has been extensively characterised. It has the properties of enhancing expression from a variety of promoters whether it is upstream or downstream of the gene and is functional in either orientation. There are a limited number of reports where the SV40 enhancer has been used in conjunction with a steroid-responsive promoter. For example Israel and Kaufman (Israel and Kaufman 1989) reported substantial dexamethasone induction of expression (up to 17 fold) constructs which combined the SV40 enhancer and adenovirus major late promoter with different copy numbers of glucocorticoid response elements derived from the mouse mammary tumour virus LTRpromotellenhancer. Wynshaw-Boris et al.

(Wynshaw-Boris, Short et al. 1986) found that the SV40 enhancer had little effect on basal level transcription or glucocorticoid-inducibility (about 6fold) of the rat phosphoenolpyruvate carboxykinase gene. The 3 to 5-fold glucocorticoid induction mediated by intragenic sequences of the rat growth hormone gene was found to be equivalent in the presence or absence of the SV40 enhancer (Birnbaum and Baxter 1986). In another case it was observed that the glucocorticoid response region of the tyrosine amino transferase gene was able to confer 2 to 3 fold glucocorticoid inducibility on the SV40 enhancer/early promoter (Grange, Roux et al. 1989). These examples indicate that continued steroid responsiveness is normally seen when the SV40 enhancer is present in conjunction with a steroid-responsive promoter.

The present inventors have combined, in various constructs, the viral SV40 enhancer with the promoter regions of two prostate-expressed, androgen-induced genes -probasin and PS. They have observed a substantial increase in the basal level of induction of the promoters without a corresponding increase in the androgen-induced level of activity. The promoter/enhancer combinations are thus now essentially androgen-independent, but maintain their specificity profile with respect to expression in prostate and non-prostate cells. Thus the probasin/SV40 enhancer combinations show substantial prostate specificity, while PSA/SV40 enhancer combinations are more promiscuous.

SUMMARY OF THE INVENTION

Accordingly in a first aspect the present invention consists in a genetic construct providing preferential expression specific tissue of a coding sequence, the construct including the following elements (1) a steroid responsive promoter from a tissue specific gene, (2) a coding sequence, and (3) an SV40 enhancer, the elements being arranged as follows:

the steroid responsive promoter from the tissue specific gene being upstream of the coding sequence and the SV enhancer being 3' of the coding sequence, or
the SV40 enhancer being upstream of the coding sequence and the steroid responsive promoter from the tissue specific gene being positioned between the SV40 enhancer and the coding sequence, or
the steroid responsive promoter from the tissue specific gene being upstream of the coding sequence and the SV enhancer being positioned within an intron within the coding sequence; wherein the promoter has a lowered level steroid responsiveness in the construct than in its native state.

In a second aspect the present invention consists in a genetic cassette comprising a steroid responsive promoter from a tissue specific gene and an SV40 enhancer and an insertion site into which a coding sequence can be inserted, the insertion site being adjacent to and downstream of the promoter, wherein the promoter has a lowered level of steroid responsiveness in the cassette than in its native state.

In this aspect of the invention the SV40 enhancer may be upstream of the tissue specific promoter or vice versa.

In a preferred embodiment of the present invention the promoter from the tissue specific gene is androgen-responsive. It is also preferred that the tissue specific gene is prostate specific.

In further preferred embodiments the tissue specific promoter is the probasin promoter, in particular Pb430 or Pb; as described herein. In another embodiment the promoter is the PSA promoter, in particular PSA630 as described herein.

In a third aspect the present invention consists in a vector including the genetic construct of the first aspect of the present invention or the genetic cassette of the second aspect of the present invention.

It is presently preferred that the vector is human adenovirus Type 5 or ovine adenovirus.

In a further aspect the present invention provides methods of treatment of cancers, in particular, prostate, bladder and breast, involving gene therapy using the constructs of the present invention. The use of these constructs enables the use of androgen-ablation treatment in combination with the expression of therapeutic genes in gene therapy for androgen-independent or androgen-dependent prostate cancer.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

FIGURE LEGENDS

Figure 1A shows a linearised map of the plasmid pCATSAT.

Restriction enzyme sites H(Hind III), Sp(Sph I), P(PstI), S(SalI), X(XbaI) and BamHI are shown; amp and ori are the plasmid ampicillin resistance gene and origin of replication respectively. The chloramphenicol acetyl transferase (CAT) and serine acetyl transferase genes are indicated as is the rous sarcoma virus promoter (RSV) and polyadenylation regions derived from the human growth hormone gene or SV40.

Figure 1B shows a number of promoter/enhancer constructs made in the pCATSAT vector. Promoter regions upstream of the CAT gene were derived from either the probasin (Pb430 or Pb286) or PSA (Psa630) genes. The SV40 enhancer is shown as a dark box, its orientation being indicated by the position of the NcoI (N) site.

Figure 2 shows the sequence of SV40 enhancer. The BstYI sites used for cloning and the unique NcoI site are underlined.

Figure 3 shows the transcriptional activity of constructs containing the Pb430 promoter in a number of cell types.

Figure 4 shows the transcriptional activity of constructs containing the 286 base pair probasin promoter in a number of cell types.

Figure 5 shows the transcriptional activity of constructs containing the 630 base pair PSA promoter in a number of cell types.

Figure 6 shows the recombinant adenovirus Ad5SVbPb430PNP. The cassette containing the SVbPb430 enhancer/promoter, the E.coli purine nucleoside phosphorylase gene (PNP) and SV40 polyadenylation region (SVpolyA) is inserted into the deleted Ela/6 region of the human adenovirus type 5. Below, in brackets, is shown the equivalent cassette with the PSA630 base pair promoter included in the virus Ad5PSAPNP.

Figure 7A shows the effect of the combination of treatment with either Ad5SVbPb430PNP (SVPb) or Ad5PSAPNP in combination with 6-MPDR or PC3 cell viability. Viability was measured as metabolic activity relative to control, without virus or drug. MOI - multiplicity of infection.

Figure 7B shows the effect of virus plus drug treatment on MRC5 cells.

Figure 7C shows the effect of virus plus drug treatment on HepG2 cells.

EXAMPLES

Plasmid Constructs.

The following plasmids combining the SV40 enhancer region in different positions and orientation with the promoter regions of the rat probasin and human PSA genes are diagrammed in Figure 1. All plasmids have been prepared using the base plasmid pCATSAT. This plasmid was derived from the plasmid pCAT-basic (Promega) by insertion into the BamHI site of a reference gene, serine acetyl transferase (SAT.) This reference reporter gene is under the control of the ubiquitously-expressed rous sarcoma virus promoter (RSV) and contains a 3' polyadenylation region derived from the human growth hormone gene. A BamHI site was re-generated as shown between the SV40 and human growth hormone 3' polyadenylation regions.

Probasin sequences from -426 (HindIII) or -286 to the SacI site at position + 28 were isolated by PCR from rat genomic DNA and cloned into pCATSAT to produce the Pb430 and Pb286 constructs, respectively. For Pb430 the PCR fragment was cloned as a SacI fragment into the SacI site of pBluescriptSK+ using the SacI site at +28 and a site incorporated into the 5' primer. It was then re-cloned as a HindIII-XbaI fragment into pCATSAT.

The Pb286 PCR fragment was cloned into pBluescriptSK+ as an EcoRI to SpeI fragment using sites contained in the 5' and 3' primers respectively; it was then cloned as an HindIII to SpeI fragment into HindIII-XbaI cut pCATSAT. The PSA630 plasmid contained sequences 5' of the PSA transcription start site from EcoRI site (-630) to the HindIII site (+6) which were isolated from human genomic DNA by PCR amplification.

SV40 enhancer sequences were isolated as a BstYI fragment from the plasmid pCAT-enhancer (Promega). The sequence of the enhancer region is shown in Figure 2. The unique NcoI site is underlined. This fragment was subcloned into the BamHI sites of the plasmids Pb430CATSAT and PSA630CATSAT to produce the plasmids Pb430CATSATSVa and Pb430CATSATSVb and PSA630CATSATSVa and PSA630CATSATSVb respectively. The a and b orientations of the enhancer are distinguished by the position of the NcoI site as indicated in Figure 1. For placement of the enhancer upstream of the Pb430 promoter the BstYI enhancer fragment was cloned into BamHI-cut pBluescriptS (Stratagene), then re-cloned as a XbaI to KpnI fragment into pUC19. It was then excised as a HindIII fragment and cloned into the HindIII site of Pb430CATSAT to give the plasmids SVaPb430CATSAT and SVbPb430CATSAT. The SV40 enhancer was cloned in front of the Pb286 promoter in the pBluescriptSK+ plasmid using BamHI site incorporated in the 5' PCR primer. The two enhancer orientations in front of Pb286 were then re-cloned as HindIII-SpeI fragments into HindIII XbaI cut pCATSAT to give plasmids SVaPb286CATSAT and SVbPb286CATSAT as shown in Figure 1.

Example 1:

A range of cell types were transfected with the Pb430CATSAT construct and plasmids containing the SV40 enhancer in either orientation either downstream of the CAT gene or immediately upstream of the probasin promoter.

Cell Types

Prostate tumour-derived cell lines used were LNCaP (obtained from Dr. L. Chung) and PC-3 (obtained from the ATCC). LNCaP cells are androgen-sensitive and express a mutated androgen receptor; PC-3, DU145 and TsuPr cells are androgen independent. Tumour cell lines derived from other tissues included the breast cancer line MCF-7 (obtained from the ATCC), the liver cell line HepG2 cells (from Dr. G. Schreiber), and the bladder cancer cell line BL13 (Russell, Ward et al. 1989). Human 293 embryonic kidney cells, adenovirus transformed, were obtained from Dr. F.

Graham. The normal lung fibroblast line MRC5 and the chinese hamster ovary line CHOK1 were obtained from C R. Holliday. LNCaP cells were maintained in T-medium (Thalmann, Sikes et al. 1996). PC-3, DU145 and TsuPr MCF7 and BL13 cells were maintained in RPMI 1640 medium containing 2mM glutamine, non-essential amino acids and 10% fetal bovine serum (FBS). HepG2 and MRC5 cells were maintained in Dulbecco's MEM containing 2 mM glutamine, 0.45% glucose and 10% FBS. CHO and 293 cells were maintained in MEM containing non-essential amino acids and 10% FBS.

Transfections

For transfection, cells were seeded at 30 to 50% confluency in 35mm dishes and transfected the following day using DOTAP (Boehringer) according to the manufacturer's protocols. Amounts of DNA and DOTAP were optimised for each cell type. Generally, 2.5 µg of DNA mixed with 15 µl of DOTAP was used to transfect one dish. Control transfections contained either plasmid (pBR322) DNA as a negative control or a standard mix of RSV-CAT and RSV-SAT plasmids as a positive control. Transfection of MRC5 cells was carried out using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Transfections of DU145 and TsuPr cells were done using another cationic lipid transfection reagent CS067 and a similar protocol provided by Dr. T. Locke (other transfection reagents such as Lipofectamine may also be used).

CAT and SAT assays:

At 44-48hr post-transfection cells were rinsed three times with PBS, and harvested by scraping into a 1.5 ml centrifuge tube and brief centrifugation. Cell pellets were resuspended in 60 µl of 0.1M Tris-HCl, pH 7.5, containing 500 µM Pefabloc protease inhibitor (Boehringer). Cells were lysed by three cycles of freezing and thawing and debris was pelleted for 5 min in a microfuge. Supernatant (20 µl) was set aside for SAT assays and the remaining extract was heated at 65°C for 10 min. Debris was again pelleted and the supernatant used for CAT assays (Sleigh 1986). Non-heated extract was used for assays of serine acetyl transferase activity. Reaction mixtures (20 µl) comprised 2 or 4 µl of cell extract, 1 mM acetyl coenzyme A and 200 µM serine containing 0.1 µCi ¹⁴C serine (Amersham, 50 mCi/ml, 150 mCi/mmol). Aliquots were removed between 20 and 120 min and reactions were stopped by heating at 95°C for 3 min. Reaction products were separated by thin layer chromatography and subjected to phosphor image analysis (Molecular Dynamics). The extent of conversion of serine to acetylated serine was determined using ImageQuant software. The ratio of RSV-CAT to RSV-SAT in the control transfection in each experiment was used to normalise promoter activities relative to the RSV promoter.

Promoter Activities

Activities of the constructs containing the Pb430 promoter and different arrangements of the SV40 enhancer in different cell types are shown in Figure 2. The promoter alone shows low activity in the LNCaP prostate cell line and negligible activity in the PC-3 line which lacks an androgen receptor. Co-transfection of the androgen receptor into PC-3 cells leads to a strong stimulation of expression, indicating the critical androgen dependence of the promoter. Among the non-prostate cell lines slight activity was seen in the MCF-7, breast, and HepG2, liver, cell lines. For a four enhancer-containing construct increased expression was seen in most cell types. The most dramatic increase was seen in the PC-3, DU145 and

TsuPr prostate cell lines where expression ranged from 10 to 60% that of the RSV promoter. This high level expression is seen in the absence of cotransfection of the androgen receptor. Indeed when the androgen receptor was co-transfected activity was generally found to be lower in PC-3 cells, perhaps reflecting competition between binding factors or stimulatory pathways. A similar effect was seen in the DU145 and TsuPr lines (data not shown). The Pb430SVa, SVaPb430 and SVbPb430 constructs showed 3.5 to 6 fold higher expression in the prostate PC-3 and DU145 cells than in any of the other cell types tested. Highest expression in non-prostate cell types was seen in the cell lines of secretory epithelial origin, the breast cancer cell line MCF-7 and the bladder cancer cell line BL13. It should be noted that the androgen receptor is expressed in the MCF-7 line. Expression in the other cell lines, 293 (kidney), MRC5 (lung fibroblast) and HepG2 (liver) was much lower, at least 20 fold, for example, for the SVbPb430 construct. Other experiments have shown that the Pb430 promoter can also be activated by

AR gene co-transfection in most non-prostate cell lines. Similarly to their function in PC-3 cells the Pb430/SV40 enhancer combinations show no or much reduced requirement for AR gene co-transfection. Thus the probasin promoter/SV40 enhancer combinations provide for substantial specificity for expression in the PC-3 androgen-independent prostate cells relative to other cell types. The lack of requirement for the presence of androgen receptor for high level expression also provides for a significant improvement in utility compared to the probasin promoter alone, as these combinations will be active in androgen-independent cancer cells and can be used while continuing androgen ablation therapy designed to eliminate androgen-dependent tumour cells.

Example 2

A range of cell types were transfected with the Pb286CATSAT construct and plasmids containing the SV40 enhancer in either orientation either immediately upstream of the Pb286 promoter.

Results of transfections are shown in Figure 4. As for the Pb430 promoter, the Pb286 promoter is very poorly expressed in PC-3 cells, but is highly expressed when co-transfected with the androgen receptor. Again, combination of the Pb286 promoter with the SV40 enhancer are highly expressed in PC-3 cells in the absence of androgen receptor. For the SVb enhancer orientation expression was 70% of that seen in the presence of the androgen receptor, while for the SVa orientation expression was reduced in the presence of the AR (similarly to the Pb430 constructs). While expression was higher in PC-3 cells than in non-prostate cell types, the specificity of expression was less than that seen for the Pb430 constructs. The effect of the enhancer is thus similar to that seen for the Pb430 constructs in bypassing the requirement for androgen induction for expression from the promoter.

Example 3.

Reduced AR requirement for expression from PSA promoter constructs incorporating the SV40 enhancer.

Expression from promoter constructs PSA630, PSA630SVa and

PSA630SVb was examined in a number of cell lines following transfection alone or in combination with the AR gene. Results are shown in Figure 5.

Expression from the PSA630 promoter was found to be significantly increased in the presence of AR in all cell types studied, though the level of induction varied considerably. Constructs containing the SV40 enhancer in either orientation 3' of the CAT gene showed substantially less enhancement of expression in the presence of AR in all cell types. The effect of AR ranged from three fold induction to two fold repression in different instances. The only exception was in MCF-7 cells where the AR enhanced expression by 5.8 and 4.3 fold for the SVa and SVb constructs respectively compared to tenfold in the absence of the enhancer. While these PSA630based constructs show poor prostate specificity the effect of the SV40 enhancer on expression from this androgen-responsive promoter is similar to that on the Pb promoters in that expression becomes substantially androgenindependent.

Example 4

The promoter cassette SVbPb430 was excised from pSVbPb430CATSAT as a Sall to XbaI fragment and blunt-ended. It was recloned in front of the E.coli purine nucleoside phosphorylase (PNP) gene in the plasmid pXCX3/PSAPNPase (Lockett et al. 1997) which had been cut with KpnI and HindIII, blunt-ended and re-ligated to remove the PSA promoter and subsequently cut with SpeI and blunt-ended. This was then used to construct, via recombination, a recombinant human Type 5 adenovirus carrying the PNP gene under the control of the SVbPb430 regulatory sequences (Figure 6). Genetic manipulation and virus construction was done as described Lockett et al. (1997). The PNP gene converts the pro-drug 6-methyl purine 2-deoxyriboside to the toxic product 6-methyl purine. 6-methyl purine can kill cells through incorporation into both RNA and DNA and because it is non-charged it can diffuse between cells and kill cells which are not expressing the enzyme (the "bystander" effect).

The activity and specificity of the virus Ad5SVbPb430PNP in mediating pro-drug dependent cell killing was compared with a virus

Ad5PSAPNP in which the expression of the PNP gene was under the control of the promoter of the prostate specific antigen (PSA) gene. Cells were seeded at approximately 105 per well into 16 mm wells (24 well plates) and 24 hr later viruses were added at ratios ranging from 1 to 200 plaque forming units (pfu) per cell (see Figure 7). Cells were incubated either in the presence of virus alone, virus plus 50 μ M 6-MPDR, or 6-MPDR alone. Cell growth and viability was determined after six days by measurement of metabolic activity using an Alamar Blue assay. No effect of drug alone was observed. At higher doses of virus some effect of virus alone was seen and in Figure 7 the cell killing effect is corrected for any virus alone effect.

It can be seen that Ad5SVbPb430PNP is substantially more effective in killing the prostate PC-3 cells than Ad5PSAPNP; an equivalent suppression was seen at virus inputs at least 10 fold lower. The same differential in killing was not seen for the non-prostate cells MRC-5 and HepG2; the two viruses showed equivalent suppression of HepG2 cells, while significant reduction in metabolic activity of MRC-5 cells following infection with Ad5SVbPb430PNP was only seen at the highest virus level tested (200 pfu).

Virus inputs required for equivalent cell killing were 5 to 10 fold higher for the non-prostate cells than for the PC-3 prostate cell line.

Thus, in the context of virally-delivered enzyme prodrug therapy, the SVbPb430 promoter provides for substantially improved activity in prostate cells compared with the PSA promoter and shows significant selectivity for prostate cells.

As will be appreciated from these Examples these promoter/enhancer combinations are new and have much greater utility than the original promoters in that they can be used to control expression of genes in a prostate-specific manner in both androgen-responsive and androgen-independent prostate cells and in the absence of androgens (in cancer patients undergoing androgen-ablation treatment). This is of particular importance for example in the expression of therapeutic genes in gene therapy for androgen-independent prostate cancer.

As will be understood the constructs of the present invention will also be useful where patient has been treated surgically (castration) or with inhibitors of androgen synthesis or action. Specifically such androgen blockade therapy can be continued without substantially diminishing the activity of these promoters.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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